HEPATOZOON CF. *TERZII* (SAMBON & SELIGMAN, 1907) INFECTION IN THE SNAKE *BOA CONSTRICTOR CONSTRICTOR* FROM NORTH BRAZIL: TRANSMISSION TO THE MOSQUITO *CULEX QUINQUEFASCIATUS* AND THE LIZARD *TROPIDURUS TORQUATUS*

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Summary:

Specimens of *Hepatozoon*-infected *Boa constrictor constrictor* were obtained from localities in Pará State, north Brazil. Gametocytes in erythrocytes of the peripheral blood measured 10 × 2.5-16.2 × 3.7 µm. They were similar to those described as *Haemogregarina terzii* by Sambon & Seligmann (1907) in *B. c. constrictor*, in that they did not distort the infected erythrocyte, and their dimensions approximated those given by Carini (1947). Lungs and liver of infected snakes contained actively dividing meronts of a single type, and cysts containing two to six cystozoites were also present in the liver. Our initial feeding of *Culex quinquefasciatus* on infected snakes consistently resulted in a heavy death-rate of the engorged mosquitoes, with only a few surviving till the 9th day post feeding. These contained numerous oocysts which were undivided or in early stages of division. A fifth and final experiment, however, provided a few mosquitoes surviving up to 21 days post infection (dpi), and these contained fully sporulated oocysts measuring 190-200 µm in diameter and containing over 60 sporocysts of 19-30 µm in diameter. The number of sporozoites in each sporocyst was estimated as approximately 50. The nature of the parasite's sporogonic cycle in the mosquito thus justifies inclusion of this haemogregarine in the genus *Hepatozoon*. Two wild-caught specimens of the lizard *Tropidurus torquatus* were fed with mosquitoes containing fully developed oocysts (21 dpi). When sacrificed, three months later, large numbers of dizoic, tetrazoic and hexazoic cysts were demonstrated in their livers. Cystozoites released from these cysts were shown to possess a conspicuous refractile body.

KEY WORDS : *Hepatozoon* cf. *terzii*, *Boa constrictor constrictor*, *Tropidurus torquatus*, *Culex quinquefasciatus*, life-cycle, Brazil.

Résumé : INFECTION À *HEPATOZOON* CF. *TERZII* (SAMBON & SELIGMAN, 1907) DU SERPENT *BOA CONSTRICTOR CONSTRICTOR* DU NORD DU BRÉSIL : TRANSMISSION AU MOUSTIQUE *CULEX QUINQUEFASCIATUS* ET AU LÉZARD *TROPIDURUS TORQUATUS*

Des Hepatozoon *ont été prélevés sur* Boa constrictor constrictor *dans l'État de Pará au nord du Brésil. Les gamétocytes érythrocytaires du sang périphérique measurent 10 × 2.5-16.2 × 3.7 µm. Ils ressemblent à ceux d'*Haemogregarina terzii *décrits par Sambon et Seligmann (1907) chez* B. c. constrictor, *par le fait qu'ils ne déforment pas les érythrocytes infectés, et leurs mensurations sont proches de celles données par Carini (1947). Les poumons et le foie des serpents infestés contiennent des mérontes en phase de division active ; des cystes renfermant de deux à six cystozoites sont également présents au niveau du foie. Les premiers repas sanguins de* Culex quinquefasciatus *sur les serpents infectés ont été suivis d'une mortalité importante des moustiques, avec seulement quelques survivants au neuvième jour suivant l'engorgement. Ceux-ci renfermaient des oocytes non divisés ou en phase de division débutante. Cependant, une cinquième et dernière expérience a permis d'observer quelques moustiques survivant au 21ème jour après l'infection, lesquels étaient porteurs d'oocytes sporulés de 190-200 µm de diamètre et contenant plus de 60 sporocytes de 19-30 µm de diamètre. Le nombre de sporozoites dans chaque sporocyte étant estimé à environ 50. Ainsi, la nature du cycle sporogonique du parasite chez le moustique justifie l'inclusion de cet haemogregarine dans le genre* Hepatozoon. *Deux lésards* Tropidurus torquatus *sauvages ont été nourris avec des moustiques porteurs d'oocytes entièrement développés (21ème jour après infection). Trois mois plus tard, un grand nombre de cystes – dizoique, tetrazoique et hexazoique – a été observé au niveau du foie de ces lésards. Les cystozoites résultant de ces cystes ont révélé un corps réfringent remarquable.*

MOTS CLÉS : Hepatozoon *cf.* terzii, Boa constrictor constrictor, Tropidurus torquatus, Culex quinquefasciatus, *cycle biologique, Brésil.*

INTRODUCTION

Boas (*Boa constrictor constrictor*) are common hosts of *Hepatozoon* species (Apicomplexa: Adeleina: Hepatozoidae). Of the two parasites described in this snake from Brazil, *H. juxtanuclearis* (Carini, 1947) induces hypertrophy and distortion of the infected erythrocytes while the other, *H. terzii* (Sambon & Seligmann, 1907), does not. The species of *Hepatozoon* commonly infecting boas in Pará State, north Brazil, does not distort the host erythrocyte and this suggests that the parasite might be conspecific with *H. terzii*.

Accumulating data suggests that species of *Hepatozoon* developing in mosquitoes may not be too fastidious in their choice of vector hosts. They have been shown to complete their sporogonic cycle in a number of common laboratory species, which are not necessarily their natural vectors, such as *Culex pipiens* (Bashtar

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et al., 1984, 1991), *C. quinquefasciatus* (Mackerras, 1962; Lainson *et al.*, 2003), *Aedes aegypti* (Lowichick *et al.*, 1993; Lainson *et al.*, 2003), *Ae. togoi* (Ball *et al.*, 1969) and *Anopheles stephensi* (Landau *et al.*, 1972). In the present communication we describe developmental stages of *H.* cf. *terzii* in its natural vertebrate host *B. c. contrictor*, and in experimentally infected mosquitoes and the lizard *Tropidurus torquatus*.

MATERIALS AND METHODS

Hepatozoon-infected boas were obtained from several localities in Pará, north Brazil. Blood was obtained by clipping the tip of the tail or by cardiac puncture, and thin films were rapidly air-dried, fixed in absolute methyl alcohol and stained by Giemsa's method. Tissue stages of the parasite, from two sacrificed snakes, were studied in dab smears fixed and stained as for the blood films, and in histological sections of material fixed in 10 % buffered formalin. The mosquitoes were from a laboratory-bred strain of *C. quinquefasciatus*, originating from Belém and maintained at an ambiant temperature of 24-26° C. In each of five attempts to obtain complete sporogony of the haemogregarine, approximately 50 mosquitoes were allowed to feed on the infected boa overnight, after which the snake was removed from the cage. Guts of fed mosquitoes were dissected out and examined, by direct light-microscopy, 24 h and 2, 6, 7, 9 days post infection (dpi) and, on the rare occasions when they survived, 21 dpi. Smears for staining were prepared from the guts at 24 h and 2 dpi, and guts at 6, 7, and 9 dpi were processed for histology.

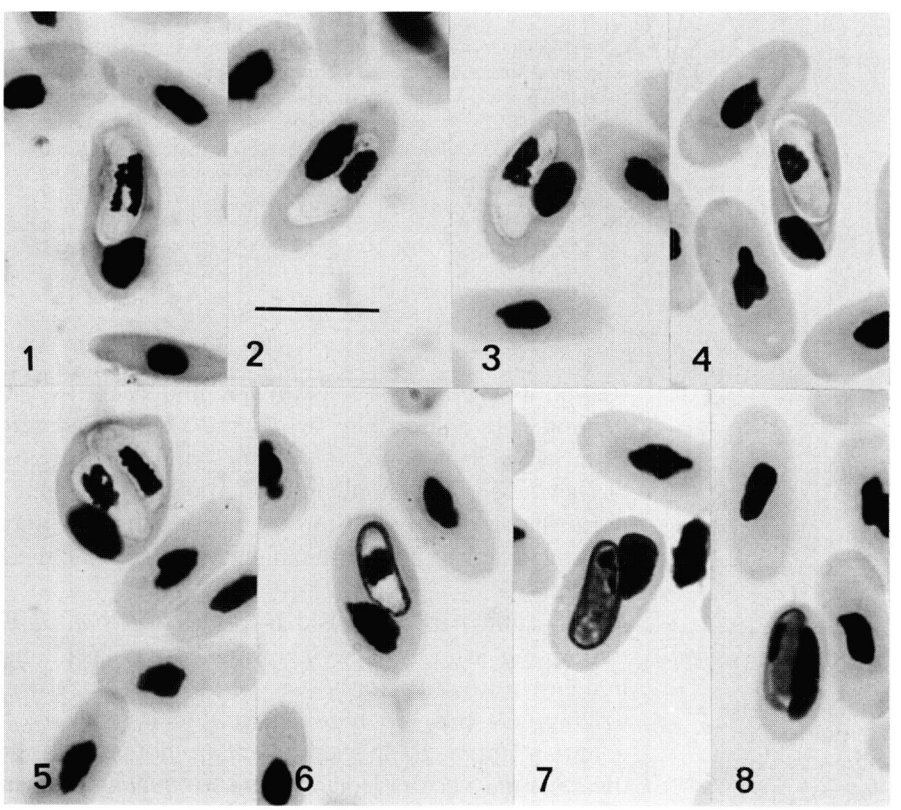
Four mosquitoes with fully sporulated oocysts were force-fed to each of two wild-caught iguanid lizards, *Tropidurus torquatus*, which were sacrificed and examined for infection three month later.

Infected snake, lizard and mosquito tissues were either fixed in buffered 10 % formalin and embedded in GMA, or fixed in buffered glutaraldehyde and embedded in "Agar" 100® medium (both resins from Agar Scientific Ltd, Stansted, UK). Sections were cut at 2 µm, with a glass knife microtome, and stained either with haematoxylin and eosin or with toluidine blue. Measurements are given in µm as means, followed by the range in parentheses and the number of parasites measured (n).

RESULTS

Gametocytes in the peripheral blood (Figs 1-8)

Gametocytes are located only in the erythrocytes: they are elongate and have a mean measurement of 12.3 × 4.3 (10 × 2.5-16.2 x 3.7), n = 50. In some erythrocytes the host-cell nucleus is forced to one side, while in others it is displaced to one end.



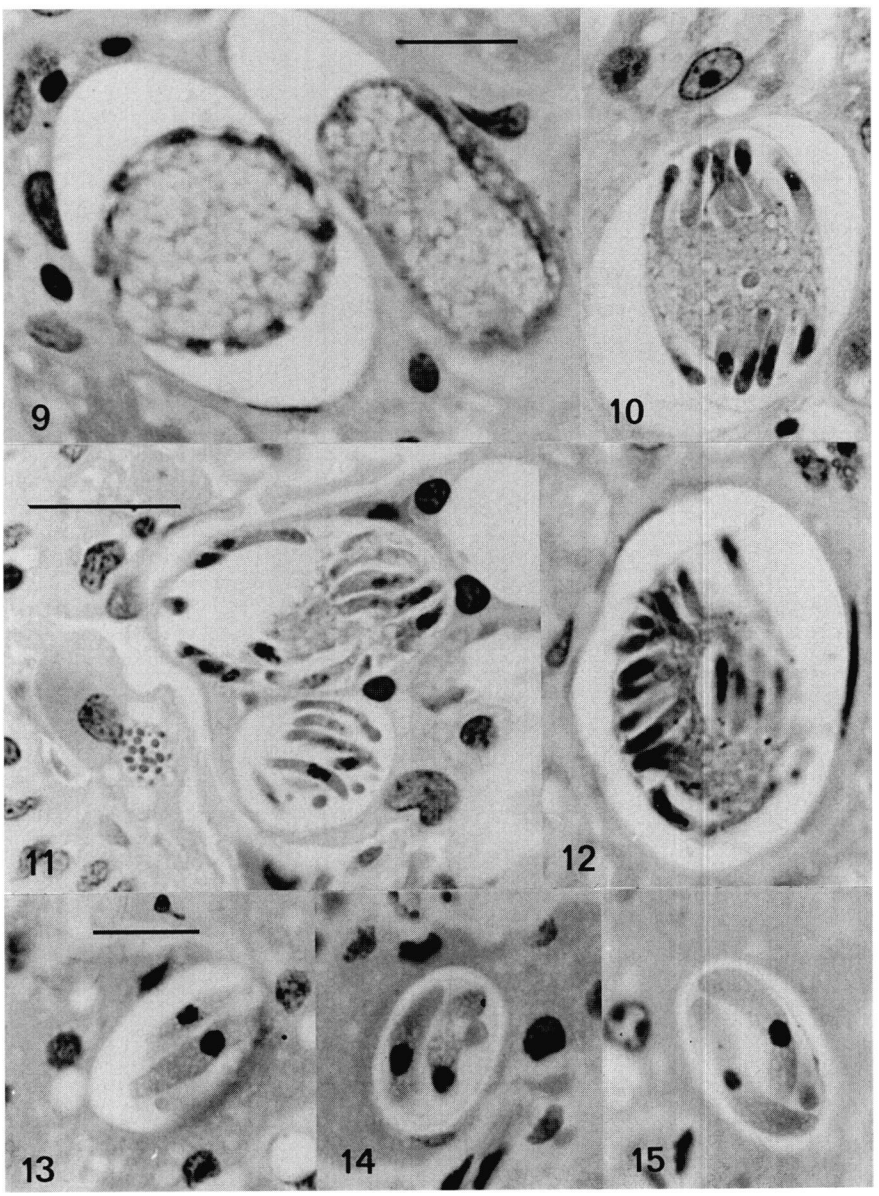
Figs 1-8. – Giemsa-stained blood film of the snake *Boa c. constrictor* infected with *Hepatozoon* cf. *terzii*. Figs 1-6. Mature, encapsulated gametocytes in erythrocytes: double infection in Figure 5. Figs 7-8. Heavily stained gametocytes, presumably immature and without a fully developed capsule. Bar = 10 µm for all figures.

Mean size of infected erythrocytes did not differ significantly from that of non-infected ones (17.9 ± 2.9 × 8.4 ± 2.1, n = 15 *versus* 19.4 ± 1.0 × 8.5 ± 1.0, n = 10; t-test, df = 23). Some of the erythrocytes with gametocytes positioned alongside the host-cell nucleus were, in fact, smaller than non-infected cells. Others, with the nucleus displaced to the end of the cell were sometimes of slightly increased length (up to 22-24 × 6.6-8.8). The nuclei forced to one side of the erythrocytes were usually flattened and, therefore, of increased length but decreased width: the difference was 9.9 ± 1.1 × 2.9 ± 0.6 for infected erythrocytes, *versus* 5.5 × 2.2-3.3, n = 6 for non-infected cells. In cells with the nucleus displaced to the end, the difference was 7.1 ± 1.2 × 4.1 ± 1.9 *versus* 5.5 × 2.2-3.3, n = 7. The latter difference is not significant by the t-test. Not surprisingly, occasional erythrocytes harbouring two gametocytes were considerably enlarged, and rounded in shape (Fig. 5)

The gametocytes showed two distinct staining reactions in the same blood film: the cytoplasm of some was colourless (Figs 1-6), while that of others stained a deep red (Figs 7-8). The colourless forms are probably older gametocytes which have developed a thicker and more stain-resistant capsule.

Development in the viscera of *B. c. constrictor* (Figs 9-15)

Both the liver and lungs contained actively dividing meronts of a single type: a few were also seen in the *lamina propria* of the digestive tract, but none were detected in the kidneys. In histological sections, undivided meronts, and others with 9-15 peripherally disposed nuclei (Fig. 9) measured 26 ± 5.7 × 21.3 ± 4.9, n = 10



Figs 9-15. – *Hepatozoon* cf. *terzii* in the snake *Boa. c. constrictor*. Fig. 9. Histological section of lung, showing undivided meronts with peripherally located nuclei. Figs 10-12. Lung sections with dividing meronts and separated merozoites. Figs 13-15. Liver sections, showing tetrazoic cysts and contained cystozoites. Haematoxylin and eosin staining. Bar in Figure 9 = 15 µm, and also serves for Figures 10 and 12: bar in Figure 11 = 15 µ: bar in Figure 13 = 10 µm and serves for Figures 14 and 15.

(19.8 × 17-35.2 × 30.8). Cross-sections of segmented meronts producing 12-31 merozoites (Figs 10-12) were 32.1 ± 4.6 × 20.2 ± 2.4, n = 5 (26.4 × 19.8-37.4 × 24.2). Probably as a result of shrinkage during fixation and processing, meronts appeared to be within a large vacuole measuring 46.6 ± 6.8 × 24.2 ± 6.6, n = 8 (Figs 9-12).

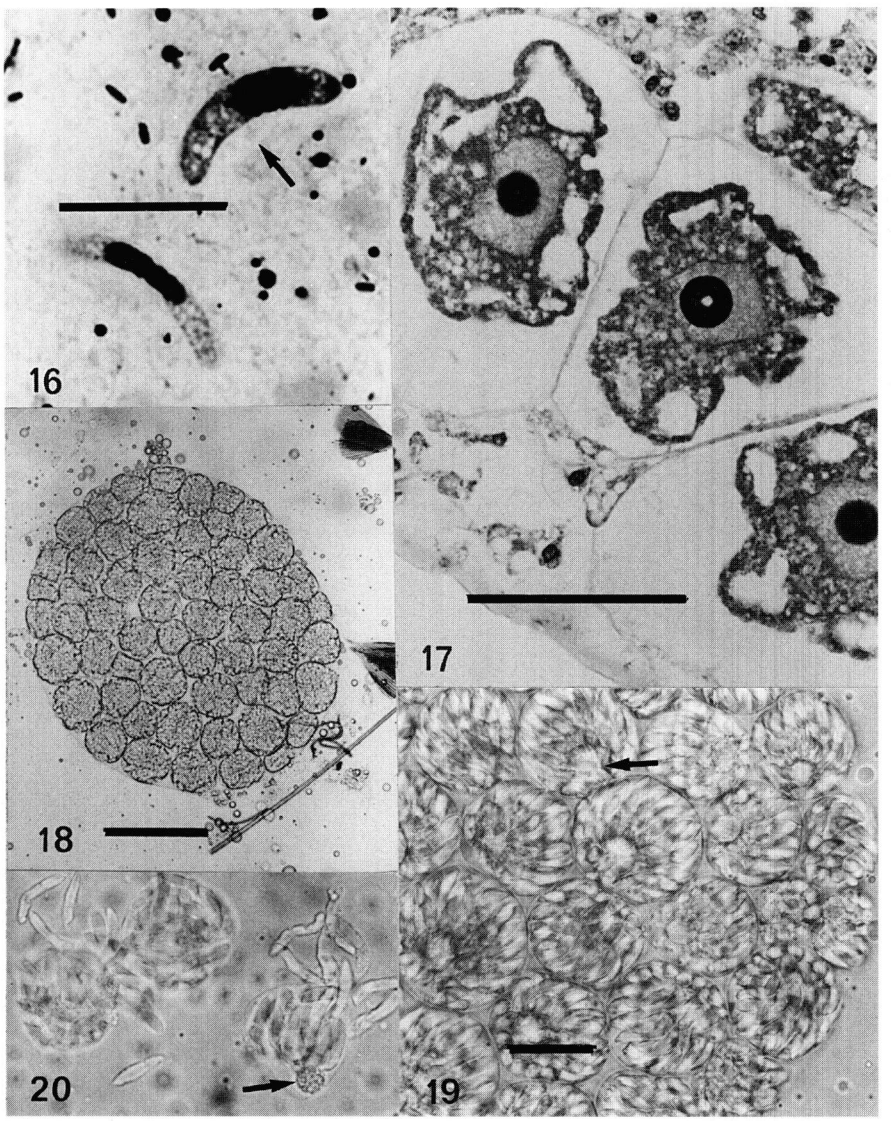
Cysts containing two, four or, rarely, six cystozoites were found predominantly in sections of the liver (Figs 13-15). Dizoic and tetrazoic cysts measured 19 ± 4 × 13.8 ± 2.3, n = 11 (13.2 × 11-28.6 × 17.6): hexazoic cysts were not measured. Cystozoites measured 17.6 ± 3.3 × 3.65 ± 0.50, n = 6 (14.3 × 4.4 to 22 × 3.3). No refractile bodies could be detected in them, as seen in histological sections (Fig. 15).

Experimental infection in *Culex quinquefasciatus* (Figs 16-20)

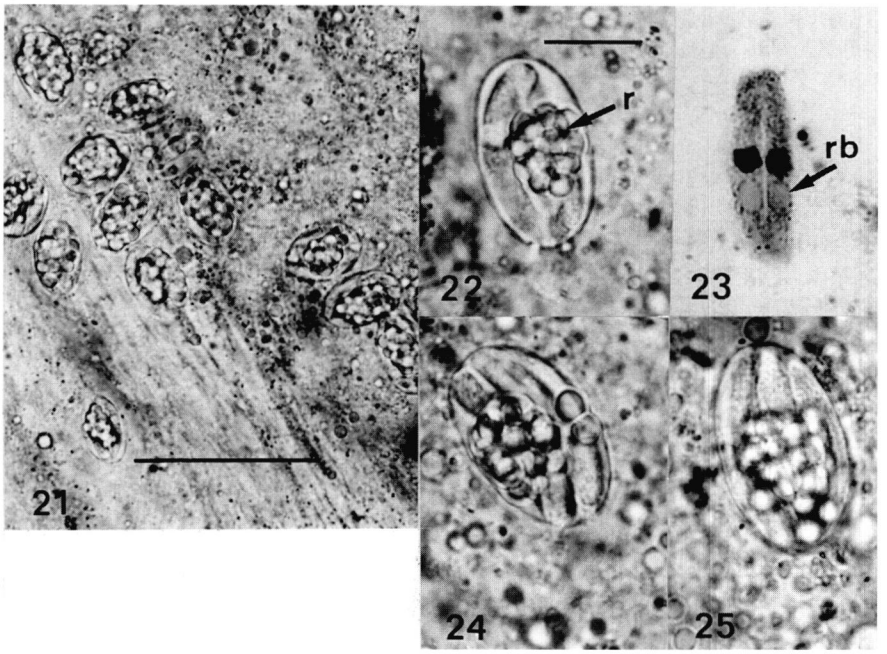
During our first four trials, there was a high death-rate among *C. quinquefasciatus* fed on infected boas, leaving no survivors after 9 dpi. The blood-meal appeared to form a hard clot, distending the intestine and distorting the insect's shape. Movement of the mosquitoes became restricted and examination of the dissected gut and haemolymph often revealed a heavy bacterial infection. In a final attempt, however, a few (10/50) did survive up to 21 dpi, enabling a study of the mature oocyst, sporocysts and differentiated sporozoites.

Stained smears of a few mosquitoes dissected at 24 hpi showed abundant, slim extracellular gametocytes (Fig. 16). No evidence of fertilization could be detected in these preparations or in others made at 2 dpi, although some stouter and almost spherical forms probably represented the rounding-up process of macrogametocytes prior to syngamy.

Sections of mosquito intestines at 6 and 7 dpi showed large numbers of heavily vacuolated, uninucleate oocysts of approximately 45 in diameter in the haemocoel, atta-



Figs 16-20. – *Hepatozoon* cf. *terzii* in the mosquito *Culex quinquefasciatus*. Fig. 16. Extracellular gametocytes in a Giemsa-stained smear of the gut 24 hours following the blood-meal: the stouter form (arrowed) is probable a macrogametocyte beginning to round up prior to syngamy. Bar = 10 µm. Fig. 17. Developing, uninucleate oocysts in the haemocoel of a mosquito seven days after the blood-meal: histological section stained with haematoxylin and eosin: bar = 50 µm. Fig. 18. Fully sporulated oocyst in a fresh preparation of a dissected mosquito, 21 days after the blood-meal: bar = 50 µm. Figs 19, 20. Enlarged view of living, intact and ruptured sporocysts: bar = 20 µm. Note conspicuous residual bodies (arrowed).



Figs 21-25. – Cysts and contained cystozoites in the liver of the lizard *Tropidurus torquatus*, three months after feeding the animal with *Culex quinquefasciatus* heavily infected with mature oocysts of *Hepatozoon* cf. *terzii* from *Boa c. constrictor*. Fig. 21. Low power view of a fresh, squash preparation to show the very large number of cysts: bar = 50 µm. Figs 22-25. High power view of living cysts, showing cystozoites and prominent residual body (r): bar = 10 µm. Fig. 23. Freed, Giemsa-stained cystozoites, each with a single refractile body (rb): same magnification as Figure 22.

ched to the gut surface (Fig. 17). At 9 dpi, uninucleate oocysts were still present in some mosquitoes, while in others they were seen to be in early stages of nuclear division (Paperna & Lainson, 2003).

At 21 dpi, the haemocoel of dissected mosquitoes contained fully sporulated oocysts measuring from 190-200 in diameter (Fig. 18), and containing 60 or more spherical sporocysts. These were packed with from 20-50 elongate sporozoites approximately 12-15 long and budded off from a residual body of 5-10 in diameter (Figs 19, 20).

Transmission of *H.* cf. *terzii* to the lizard *Tropidurus torquatus*

The two lizards force-fed with mosquitoes containing mature oocysts were sacrificed three months later. Fresh squash preparations of liver tissue showed enormous numbers of cysts containing 2, 4 or 6 cystozoites and a bulky residual body of large globules (Figs 21-25). The living cysts measured 20 × 10-12 (dizoic) to 23 × 15 (tetra or hexazoic). Two freed cystozoites, in a Giemsa-stained liver smear, measured approximately 20 × 3 and contained a single refractile body (Fig. 23)

DISCUSSION

Gametocytes of the parasite described as *Haemogregarina terzii* by Sambon & Seligmann (1907) in *B. c. constrictor* did not increase the size of the host erythrocyte, whereas both of the haemogregarines from *B. c. constrictor* subsequently described as *Haemogregarina juxtanuclearis* (Carini, 1947) and *Hepatozoon fusifex* Ball, Chao & Telford, 1969 induced extreme hypertrophy of the host cell, particularly at the final stage of their development. For this reason, and the fact that Carini's measurements for *H. terzii* (12-14 × 2.3-3) are within the range of measurements of the gametocytes in the present study, we feel we are dealing with the same organism. Furthermore, transference of the parasite to the genus *Hepatozoon* by Smith (1996), albeit without evidence from its life-cycle, is amply substantiated by the present description.

In boas infected with *H. fusifex*, merogony is most commonly found in the lungs, to a lesser extent in the liver, spleen, kidneys, heart and brain, and with distinctive micro- and macro-meronts (Ball *et al.*, 1969). In our study the lungs and liver were the principal site of merogony for *H.* cf. *terzii* in that order and we found no meronts in other organs except for a few in the *lamina propria* of the small intestine. All segmented meronts seen were of the same type and, in view of the advanced stage of the infections, are regarded as micromeronts producing micromerozoites which are destined to be gametocytes. Cysts with cystozoites were not mentioned in the description of *H. fusifex*, but illustrations in the description of *H. juxtanuclearis* appear to show cysts containing cystozoites in the liver (Pessôa, 1967).

As noted by Smith (1996), there is a high degree of plasticity for many morphological and developmental features among the species of *Hepatozoon*, including those of their sporogonic development in the invertebrate host. This, and relatively low vertebrate and

invertebrate host specificity of some of these parasites, at least under experimental conditions (Landau *et al.*, 1972; Ball *et al.*, 1967; Booden *et al.*, 1970), often makes species definition a difficult task. It does seem that there are exceptions to a loose invertebrate host-specificity: thus, we found *Culex quinquefasciatus* to poorly support infection with *H.* cf. *terzii,* and although Smith *et al.*, (1994) successfully infected *C. pipiens* and *C. teritans* with *H. sipedon,* they failed to infect *Aedes aegypti.* A similar incompatability has been shown in attempts to infect *Aedes aegypti* with haemogregarines from the snake *Coluber constrictor,* whereas the same mosquito was shown to be susceptible to infection with haemogregarines from another snake, *Nerodia fasciata* (Wosniak & Telford, 1991). In the case of mosquito hosts it would appear that their survival following a blood-meal on a *Hepatozoon*-infected animal may be seriously compromised both by difficulties in digesting an unaccustomed type of blood, resulting in bacterial proliferation, and the ingestion of an excessive number of haemogregarines (Mackerras, 1962 and our present findings). The danger of basing species definition on morphology of the blood forms alone has long been recognized (Ball, 1958; Ball *et al.*, 1967), due to the great variety of growth stages – from small merozoites to mature gametocytes. In addition, the fact that a given species may produce strikingly different cytopathological effects on the infected blood cell in different hosts has also to be considered (Ball *et al.*, 1967).

With regards to sporogonic stages, Ball *et al.* (1967) found that the only difference between the oocysts of *H. rarefaciens* and *H. fusifex* was the number of sporocysts they produced. We found the mature oocysts of *H.* cf. *terzii* to be somewhat smaller than those of both *H. rarefaciens* and *H. fusifex*, while the maximum number of sporocysts per oocyst was less than the number in *H. fusifex* and about that same as that in *H. rarefaciens.* The number of sporozoites per sporocyst in *H.* cf. *terzii* was estimated as approximately 50, whereas the number given for *H. fusifex* and *H. rarefaciens* was 15-35 and 13-42 respectively (see Smith, 1996).

As the lizards fed with infected mosquitoes in our study were wild-caught, we cannot be absolutely certain that the cysts found in their livers, three months later, were those of the boa parasite. The enormous numbers of cysts found in both lizards, however, strongly suggests that they were. Mosquitoes are not normally included in the diet of snakes and crocodilians, and it is now recognized that the ingestion of the infective, cystic stages of *Hepatozoon* in intermediate insectivorous hosts, such as lizards and frogs, is a major mode of transmission among snakes (Landau *et al.*, 1972; Smith, 1996) and crocodilians (Lainson *et al.*, 2003).

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